Adapter protein with a pleckstrin homology (PH) and an Src homology 2 (SH2) domain (APS) and SH2-B enhance insulin-receptor autophosphorylation, extracellular-signal-regulated kinase and phosphoinositide 3-kinase-dependent signalling

Zamal AHMED¹ and Tahir S. PILLAY²

Institute of Cell Signalling and School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

Adapter protein with a pleckstrin homology (PH) and an Src homology 2 (SH2) domain (APS) and SH2-B are adapter proteins and substrates that interact with the activation loop of the insulin-receptor (IR) kinase. These proteins are homologous and share substantial sequence similarity. We previously showed [Ahmed, Smith and Pillay, *FEBS Lett.* **475**, 31–34], for the first time, that insulin-stimulated phosphorylation of APS led to interaction with c-Cbl in 3T3-L1 adipocytes and in transfected Chinese-hamster ovary (CHO) cells. In the present study, we find that insulin stimulates the membrane translocation and phosphorylation of APS to a much greater extent than SH2-B, despite the structural similarity of these proteins. Expression of APS or SH2-B delays IR tyrosine and IR substrate (IRS) dephosphorylation. This enhancement of signalling is also observed downstream of the receptor. In control cells that lack APS,

following insulin stimulation, extracellular-signal-regulated kinase (ERK) and Akt kinase reach maximal activation and then decline to basal levels by 60 min. In contrast, in APS- and SH2-B-expressing cells, ERK and Akt kinase activation remains at peak levels at 60 min. These effects may occur because these proteins either stabilize the active conformation or prevent dephosphorylation of the IR. We therefore conclude that, despite the ability to couple to c-Cbl, APS functions as a positive regulator of IR signalling and, although SH2-B is a poor substrate for the IR, its association with the IR allows it to regulate pathways downstream of the receptor independently of its phosphorylation.

Key words: Akt kinase, c-Cbl, tyrosine kinase.

INTRODUCTION

Binding of insulin to the insulin receptor (IR) leads to autophosphorylation and activation of the tyrosine kinase [1]. Activation of the IR kinase leads to phosphorylation of a number of intracellular substrates, including the IR substrate (IRS) family and Src homology 2 (SH2)-domain-containing and collagen (SHC) proteins. IRS-1 and IRS-2 are critical for the activation of multiple signalling pathways by the IR [2,3]. Tyrosine phosphorylation of these proteins creates recognition sites for SH2-domain-containing signalling molecules that include the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). Once activated, PI3K stimulates activation of several downstream effectors, including Akt/protein kinase B and p70 S6 kinase. Numerous studies have confirmed a role for PI3K in metabolic signalling as well as in anti-apoptotic and/or mitogenic signalling mediated by insulin [4–6]. Translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane is essential for insulin-stimulated glucose transport into fat and muscle cells. The activity of PI3K is also necessary and partially sufficient for insulin-induced glucose transport (reviewed in [4]).

Phosphorylated SHC and IRS proteins also have specific recognition sites for the SH2 domain adapter protein Grb2 (growth-factor-receptor-bound protein 2), which contains a central SH2 domain and two SH3 domains. Grb2 associates with

the Ras guanine nucleotide exchange factor son of sevenless (SOS) via the C-terminal SH3 domain. In resting cells, Grb2 is associated constitutively with SOS. The IRS and SHC proteins mediate the translocation of the Grb2–SOS complex from the cytoplasm to the plasma membrane in an insulin-dependent manner. Membrane localization of SOS is sufficient for Ras activation [3]. The activation of p21^{ras} by insulin is rapid and leads to initiation of the Ras/Raf/MEK [mitogen-activated protein kinase/extracellular-signal-regulated kinase (ERK) kinase]/ERK signalling cascade. The activation peaks in the first 10 min following stimulation then falls even in the continuous presence of insulin [7]. This indicates that strict control of p21^{ras} activation is important for the growth regulation of insulin. The down-regulation of p21^{ras} is thought to occur through a negative-feedback mechanism, where activated ERK phosphorylates SOS on multiple serine/threonine residues resulting in dissociation of SOS from the Grb2 [8]. This in turn reduces the affinity of Grb2 C-terminal SH3 domains for SOS, preventing further translocation of SOS to the plasma membrane.

Previously, we and others identified members of the SH2-B/APS [adapter protein with a pleckstrin homology (PH) and an SH2 domain] adapter protein family as IR-activation-loop binding proteins that undergo insulin-stimulated tyrosine phosphorylation [9–12]. Furthermore, using APS-deficient Chinese-hamster ovary (CHO) cells, we demonstrated that APS was sufficient to

Abbreviations used: APS, adapter protein with a PH and an SH2 domain; CHO, Chinese-hamster ovary; CHO.T, IR-transfected CHO; ERK, extracellular-signal-regulated kinase; FCS, foetal calf serum; GLUT4, glucose transporter 4; Grb2, growth-factor-receptor-bound protein 2; IR, insulin receptor; IRS, IR substrate; JAK, Janus kinase; MEK, mitogen-activated protein kinase/ERK kinase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PTP, phosphotyrosine phosphatase; SH2, Src homology 2; SHC, SH2-domain-containing and collagen; SOS, son of sevenless.

¹ Present address: Department of Biochemistry and Molecular Biology, Darwin Building, University College London, Gower Street, London WC1E 6BT, U.K.

² To whom correspondence should be addressed (e-mail tpillay@nottingham.ac.uk).

recruit the proto-oncogene c-Cbl to the IR and facilitate phosphorylation of c-Cbl [13]. Recently, APS was shown to be necessary for insulin-stimulated phosphorylation of c-Cbl and GLUT4 translocation [14]. APS and SH2-B α are members of a family of structurally related adapter proteins that contain a C-terminal SH2 domain, a central PH domain and an N-terminal proline-rich region (reviewed in [15]). Other members of this family include SH2-B β , SH2-B γ and Lnk [15]. Although both APS and SH2-B share similar structural properties, in IR-transfected CHO (CHO.T) cells, endogenous SH2-B failed to recruit c-Cbl to the IR. Furthermore, APS and SH2-B were reported to show marked differences in their interaction with Grb2 [16]. Only tyrosine-phosphorylated APS interacted with Grb2, whereas the Grb2-SH2-B α interaction was constitutive and phosphorylation-independent. The ability of APS and SH2-B to bind differently to other signalling molecules, together with their differential expression patterns, suggested that they may have distinct cellular signalling functions, warranting further study. In the present paper, we report that overexpression of either APS or SH2-B in CHO.T cells results in enhanced IR autophosphorylation, ERK and Akt kinase activation. Furthermore, APS also shifts the dose response of insulin stimulation of its cognate receptor.

EXPERIMENTAL

Materials

Mouse monoclonal 9E10 anti-myc antibody was prepared from 9E10-hybridoma cells, and anti-phosphotyrosine antibody RC20H conjugated to horseradish peroxidase was purchased from Transduction Labs (Lexington, KY, U.S.A.). Rabbit polyclonal antibody directed against the IR β subunit, antibody directed against phosphorylated ERK and a rabbit anti-myc polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibody specific for phosphorylated Akt (that detects Akt phosphorylated on Ser⁴⁷³) was purchased from Promega. The anti-phosphotyrosine phosphatase α (PTP α) antibody was raised against a glutathione S-transferase fusion protein of the tandem catalytic domains. Anti-PTP1B antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Enhanced chemiluminescence detection reagents (Supersignal) were purchased from Pierce. Standard protein molecular-mass markers were purchased from Bio-Rad.

Cell culture

CHO.T cells overexpressing human IR were cultured in nutrient mixture Ham's F12 (Sigma) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 400 μ g/ml G418. Myc-tagged rat APS and SH2-B cDNAs were kindly provided by Dr David Ginty (Johns Hopkins University, Baltimore, MD, U.S.A.) and subcloned into the plasmid pIRESHygro (Clontech). CHO.T cells overexpressing rat APS and rat SH2-B, and control CHO.T-Hygro cells were selected in 800 μ g/ml Hygromycin and maintained in 400 μ g/ml Hygromycin as previously described in [13].

Cell lysis and immunoprecipitation

Cells were stimulated with 100 nM insulin for 5 min, followed by two rapid washes with PBS (140 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ and 1.47 mM KH₂PO₄) and lysed with lysis buffer containing 50 mM Hepes, pH 7.5, 1% (v/v) Nonidet P40, 1 mg/ml Bacitracin, 1 mM EDTA, 10 mM NaF, 1 mM sodium

orthovanadate, 10% (v/v) glycerol, 50 mM NaCl, 1 mM PMSF and 100 μ l of Protease Inhibitor Cocktail Set III (Calbiochem). The detergent-insoluble materials were sedimented by centrifugation at 20000 *g* for 15 min at 4 °C. Whole-cell lysates (0.5 mg) were used for each immunoprecipitation.

Quantification of Western (immuno)blots

Chemiluminescent images were analysed by densitometry on a Bio-Rad Chemidoc system. Prior to loading, protein levels in cell lysates were assessed using a Bio-Rad Protein Assay (Coomassie Blue-based) and then equal amounts of protein were loaded. Subsequently, the blots were probed with the respective antibodies to confirm equivalent loading and protein expression and this was used to normalize densitometry values.

Immunofluorescence

CHO.T-APS and CHO.T-SH2-B cells were plated on coverslips and then serum-starved for 16 h before insulin stimulation with 100 ng/ml insulin for 1 min. The cells were then rapidly fixed in 4% (w/v) formaldehyde prior to blocking with serum. The cells were immunostained with 9E10 anti-myc-FITC antibody and then visualized using confocal microscopy on a Zeiss LSM 510. The excitation wavelength was 488 nm and the emission filter was 505–530 nm.

RESULTS

APS sensitizes cells to insulin stimulation of IR phosphorylation

The effect of APS expression on insulin-stimulated phosphorylation was investigated using the previously established CHO.T-Hygro and CHO.T-APS cells. Insulin-stimulated IRS-1 tyrosine phosphorylation was compared between these two cell lines. Serum-starved cells were stimulated with various concentrations of insulin for 5 min before lysis. Cell lysates were analysed by Western blotting using an anti-phosphotyrosine antibody. In CHO.T-Hygro cells, the extent of IRS phosphorylation at 10 ng/ml insulin was equivalent to that seen in CHO.T-APS cells at 1 ng/ml insulin. APS phosphorylation was also observed at 1 ng/ml insulin in addition to IRS-1 (Figure 1). These results suggest that a lower concentration of insulin was required for insulin-stimulated IRS-1 phosphorylation in cells that express APS. The expression of IRS proteins or the IR β subunit was unchanged between the cell lines (Figure 1a, lower panels).

In order to exclude a secondary effect due to the up-regulation of tyrosine phosphatases, the tyrosine phosphatase levels in cells were artificially elevated using sodium orthovanadate (100 μ M) and then the vanadate was removed. The cells were then analysed at different time points to determine if there were any marked differences in the rate of decay of tyrosine phosphorylation (Figure 1c). Vanadate increased the tyrosine phosphorylation of a 60 kDa protein that decayed at a similar rate in both cells. We also analysed the expression of two candidate tyrosine phosphatases that regulate insulin signalling, PTP1B and PTP α . There was no change in the expression of PTP α , but there was a 50% increase in the expression of PTP1B (Figure 1d).

Decay of tyrosine phosphorylation of the IR β subunit and IRS proteins following insulin stimulation

It has been reported that the overexpression of SH2-B β with Janus kinase 2 (JAK-2) enhances the phosphorylation of JAK-2 and other cellular proteins [17]. CHO.T cells overexpressing either APS or SH2-B were used in order to determine if a similar phenomenon exists in the IR signalling pathway. Overexpression

Figure 1 APS expression sensitizes cells to insulin-stimulated phosphorylation

(a) Serum-starved cells were stimulated with the indicated concentrations of insulin for 5 min and detergent-soluble cell lysates were immunoblotted using anti-phosphotyrosine antibody. Cell lysates were normalized by protein assay prior to loading, and protein expression and loading was assessed by blotting aliquots of the samples for IR and IRS-1. (b) Changes in IRS phosphorylation in CHO.T-Hygro (solid line) and CHO.T-APS (broken line) cells, as determined by densitometry. (c) Analysis of tyrosine dephosphorylation in CHO.T-Hygro and CHO.T-APS cells. Cells were incubated with 100 μ M sodium orthovanadate for 30 min and then washed with PBS and re-incubated in medium for the indicated time periods to examine the rate of dephosphorylation. Molecular masses (in kDa) are indicated to the left of the gel. (d) Immunoblot analysis of two candidate tyrosine phosphatases PTP α and PTP1B.

of APS or SH2-B in CHO.T cells did not alter basal IR autophosphorylation or the tyrosine phosphorylation of other cellular proteins (Figures 2a and 2b, lanes 5). However, following acute insulin stimulation, the decay of the IR autophosphorylation was affected by overexpression of either adapter protein. Cells were incubated with insulin for 5 min, followed by rapid washing to remove free insulin, and allowed to recover for designated time periods in serum-free medium before lysis. The cell lysates were resolved by SDS/PAGE (7.5% gels) and immunoblotted first with anti-phosphotyrosine antibody (Figures 2a and 2b, upper panels) and then stripped and reprobed with anti-IR antibody (Figures 2a and 2b, lower panels). The tyrosine dephosphorylation of the IR in CHO.T-APS and CHO.T-SH2-B cells was compared with the control (CHO.T-Hygro) cells, which expressed only the Hygromycin-resistance gene. In control cells, IR phosphorylation was still detected at 15 min after insulin stimulation and reached basal levels by 60 min (Figures 2a

and 2b, lanes 1–4). In contrast, 3-fold higher IR phosphorylation was detected in CHO.T-APS and CHO.T-SH2-B cells 15 min after insulin stimulation (Figures 2a and 2b, lanes 6) and persisted up to 60 min after insulin stimulation (Figures 2a and 2b, lanes 7 and 8). Along with the IR phosphorylation, persistent phosphorylation of IRS and other cellular proteins was also detected up to 60 min after insulin stimulation (Figures 2a and 2b, lanes 7 and 8). Although there was a gradual decline in phosphorylation, these did not reach the basal levels observed in control CHO.T-Hygro cells.

Insulin-stimulated ERK activation following expression of APS and SH2-B

IR activation results in the activation of the ERK cascade that plays an essential role in the insulin-induced mitogenesis. To

Figure 2 Dephosphorylation of the IR and IRS proteins following insulin stimulation in CHO.T cells expressing APS and SH2-B

Serum-starved cells were stimulated with 100 nM insulin for 5 min, washed twice with PBS and left for the indicated time periods in serum-free medium. The whole-cell lysates were quantified by protein assay. Protein (50 μ g) was loaded in each lane and immunoblotted with anti-phosphotyrosine antibody RC20 (upper panels), stripped and reprobed with anti-IR antibody (lower panels). Molecular masses (in kDa) are indicated to the left of the gels. **(a)** Comparison of IR dephosphorylation between CHO.T-APS cells and CHO.T-Hygro cells. **(b)** Comparison of IR dephosphorylation between CHO.T-SH2-B cells and CHO.T-Hygro cells. The data represent three independent experiments. **(c)** Analysis of the changes in IR phosphorylation by densitometry.

investigate the effect of APS and SH2-B overexpression on ERK signalling. ERK activation was compared following acute insulin stimulation. The p21^{ras}-mediated ERK activation was detected using antibodies to phosphorylated p42 and p44 ERK. The cells were stimulated for 5 min and were then allowed to recover in serum-free medium for designated time periods and lysed. In the control CHO.T-Hygro cells, 60 min after stimulation, ERK phosphorylation returned to the basal (pre-stimulation) state (Figures 3a and 3b, lanes 4), consistent with other reports

Figure 3 Comparison of ERK activation between CHO.T-APS, CHO.T-SH2-B and CHO.T-Hygro cells

Cells were serum-starved for 12 h, stimulated with 100 nM insulin for 5 min, washed twice with PBS and left for the indicated time periods in serum-free medium before solubilizing. Protein (50 μ g) was analysed in an immunoblot using an antibody that detects phosphorylated ERK (pERK) (upper panels). Blots were stripped and reprobed with anti-ERK antibody (lower panels). Molecular masses (in kDa) are indicated to the left of the gels. **(a)** Comparison of ERK activation between CHO.T-APS cells and CHO.T-Hygro cells. **(b)** Comparison of ERK activation between CHO.T-SH2-B cells and CHO.T-Hygro cells. The data represent two independent experiments. **(c)** Changes in insulin-stimulated ERK activation, as quantified by densitometry.

[7,8,18,19]. In contrast, in APS- and SH2-B-overexpressing cells, insulin-stimulated ERK phosphorylation was 2–3-fold higher and persisted for up to 60 min after stimulation (Figures 3a and 3b, lanes 6–8). Phosphorylation of both p42 and p44 ERK was observed following insulin stimulation. However, in CHO.T-APS cells, more prominent phosphorylation of the p42 ERK was also observed (Figure 3a, lanes 6–8). The expression levels of ERK were not altered following the expression of APS or SH2-B (Figures 3a and 3b, lower panels).

Figure 4 Comparison of insulin-stimulated activation of Akt between CHO.T-APS, CHO.T-SH2-B and CHO.T-Hygro cells

(a) CHO.T-Hygro, CHO.T-APS and CHO.T-SH2-B cells were serum starved overnight, stimulated with 100 nM insulin for 5 min, then washed twice with PBS and left for the indicated time periods in serum-free media. Whole-cell lysates (50 μ g) were analysed by immunoblotting using anti-phospho-Akt antibody (upper panel), stripped and reprobed with anti-Akt antibody (lower panel) to analyse protein expression. The data represent two independent experiments. Molecular masses (in kDa) are indicated to the left of the gel. (b) Changes in insulin-stimulated Akt activation, as quantified by densitometry.

APS and SH2-B overexpression enhance Akt activation

PI3K is activated following growth-factor stimulation of cells and it is required for cell survival and metabolism. We tested whether APS and SH2-B can modulate PI3K-dependent signalling using the activation of Akt as an index. Cells were stimulated for 5 min and then insulin was withdrawn for designated time intervals before lysis. Total cellular proteins from CHO.T-APS, CHO.T-SH2-B and control CHO.T-Hygro cells were separated by SDS/PAGE (7.5% gels) and analysed by immunoblotting with a phosphorylation-specific (Ser⁴⁷³) Akt antibody. In the control CHO.T-Hygro cells, Akt phosphorylation was detected at 30 min after acute insulin stimulation, but declined by 60 min (Figure 4a, lanes 6–8). In SH2-B- and APS-overexpressing cells, Akt phosphorylation was also observed at 30 min after insulin stimulation, but in APS-expressing cells, this phosphorylation was higher than in the CHO.T-Hygro cells or CHO.T-SH2-B cells (Figure 4a, lane 3 compared with lanes 7 and 11). In contrast with CHO.T-Hygro cells, Akt phosphorylation did not decline in CHO.T-SH2-B and CHO.T-APS cells at 60 min after insulin stimulation, but remained approx. 3-fold higher (Figure 4a, lanes 5 and 12). The observation that insulin-stimulated Akt phosphorylation was more prominent in CHO.T-APS cells than the control or CHO.T-SH2-B cells suggests APS is perhaps more effective at modulating Akt phosphorylation than SH2-B. In CHO.T-APS cells, the level of Akt phosphorylation observed at 30 min was similar to the levels seen at 60 min in SH2-B cells (Figure 4a, lane 3 compared with lanes 7 and 11). Probing the cell lysates with an anti-Akt antibody showed that the expression level of Akt was unchanged between the three cell lines.

Differential phosphorylation and translocation of APS and SH2-B

Our laboratory has previously demonstrated that the adapter protein SH2-B interacts with the IR and undergoes, albeit weak, insulin-stimulated phosphorylation [9]. However, others have only demonstrated an interaction between the IR and SH2-B [10]. CHO.T-APS and CHO.T-SH2-B cells were stimulated with various concentrations of insulin and cell lysates were prepared. APS and SH2-B were immunoprecipitated from cellular proteins using a mouse monoclonal anti-myc antibody. Immunoprecipitants

Figure 5 Differential phosphorylation of APS and SH2-B

CHO.T-APS (A) and CHO.T-SH2-B (B) cells were serum starved overnight then stimulated with the indicated concentrations of insulin for 5 min and lysed. Myc-tagged APS and SH2-B were immunoprecipitated from 500 μ g of whole-cell lysates using mouse monoclonal anti-myc (9E10) antibody. The immunoprecipitates were immunoblotted first with anti-phosphotyrosine antibody RC20 (upper panels), stripped and reprobed with rabbit polyclonal anti-myc antibody (lower panels). The final concentrations of insulin are shown beneath the blots. B, basal (no insulin). Molecular masses (in kDa) are indicated to the side of the gels.

Figure 6 Comparison of APS and SH2-B as substrates for the IR at varying levels of expression

Two CHO.T-SH2-B cell clones that expressed relatively high or low levels of protein along with CHO.T-APS cells were serum starved overnight, stimulated with 100 nM insulin for 5 min, and then washed twice with PBS and solubilized. Myc-tagged APS and SH2-B were immunoprecipitated from 500 μ g of whole-cell lysates using anti-myc (9E10) antibody and analysed by Western blotting using anti-phosphotyrosine antibody (upper panel), stripped and reprobed with anti-myc antibody (lower panel) to show total immunoprecipitated proteins. Molecular masses (in kDa) are indicated to the left of the gels.

were resolved by SDS/PAGE (7.5% gels) and immunoblotted first with anti-phosphotyrosine-specific antibody and then reprobed with rabbit polyclonal anti-myc antibody. The anti-phosphotyrosine immunoblot shows that SH2-B was phosphorylated only when cells were stimulated with 100 ng/ml insulin, a maximal concentration of insulin. At this concentration of insulin, the observed SH2-B phosphorylation was weak, but detectable (Figure 5B). In contrast, APS phosphorylation was observed at 100 pg/ml insulin: 1000 times less than the required concentration for SH2-B phosphorylation. The phosphorylation of APS increased with higher concentrations of insulin (Figure 5A, lanes 2–4). Although a low level of APS phosphorylation was observed in the unstimulated cells, the insulin-stimulated increase in the level of APS phosphorylation was clearly distinguishable from the basal (Figure 5A, lane 1 compared with lanes 2–4). Therefore, these results show that, although both APS and SH2-B undergo insulin-stimulated tyrosine phosphorylation, there are marked differences in their suitability as substrates. The ability of SH2-B to enhance the IR, ERK and Akt phosphorylation without being a good substrate suggests that an interaction with the IR is sufficient for this effect, independently of its phosphorylation.

An increase in the abundance of SH2-B proteins in the intracellular pool may improve its phosphorylation due to its greater availability. CHO.T-SH2-B cells that expressed different levels of proteins were used to investigate whether greater *in vivo* SH2-B protein expression would proportionally increase its phosphorylation. Two CHO.T-SH2-B cell clones, one expressing relatively high and the other low levels of SH2-B protein, were stimulated with 100 ng/ml insulin for 5 min and cell lysates were prepared. The myc-tagged SH2-B and APS were immunoprecipitated using anti-myc antibody and immunoblotted with phosphotyrosine-specific antibody (Figure 6, upper panel). Reprobing of the membrane with anti-myc antibody shows the total immunoprecipitated proteins (Figure 6, lower panel). The two CHO.T-SH2-B cell clones showed marked differences in the total immunoprecipitated proteins (Figure 6, lower panel,

Figure 7 Translocation of APS, but not SH2-B, in response to insulin

CHO.T-APS and CHO.T-SH2-B cells were stimulated with insulin for 30 s and then APS and SH2-B were visualized by immunofluorescent staining and confocal microscopy. The arrows (upper right panel) show the typical membrane staining revealed by the translocation of Myc-tagged APS. Scale bars represent 50 μ m.

lanes 1 and 2 compared with lanes 5 and 6), but, interestingly, equivalent levels of SH2-B phosphorylation (Figure 6, upper panel, lanes 2 and 6). Therefore, from this experiment, it appears that the SH2-B phosphorylation *in vivo* does not increase with increased levels of expression.

In intact cells, insulin-stimulated phosphorylation of substrates may be affected by the ability to translocate to the cell membrane. We examined this hypothesis by immunofluorescence and confocal imaging of cells that were stimulated with insulin. Serum-starved CHO.T-APS and CHO.T-SH2-B cells were stimulated with insulin and then rapidly fixed and stained with 9E10 anti-myc antibody to visualize the myc-tagged APS or SH2-B proteins. In both CHO.T-APS cells and CHO.T-SH2-B cells, a diffuse cytoplasmic distribution of protein is observed. In CHO.T-APS cells, insulin causes the appearance of a rim of immunofluorescent staining (Figure 7), which is not observed in CHO.T-SH2-B cells. Thus insulin stimulates the translocation of APS, but not SH2-B, to the plasma membrane.

DISCUSSION

Proteins that interact with the activation-loop region of the IR have the capacity to regulate insulin signalling. Such proteins include the Grb10/Grb14 family [20], PTP1B [21–23] and the SH2-B/APS family [15]. We found that both APS and SH2-B enhanced insulin- and insulin-like growth factor-1-stimulated signalling at the level of the receptor and downstream of the receptor. Previously, we showed that APS coupled the IR to the phosphorylation of c-Cbl [13]. Recently, APS has been shown to be necessary for c-Cbl phosphorylation and GLUT4 translocation [14]. Thus the overall role of APS in insulin signalling appears to be a positive one in contrast with that observed with platelet-derived-growth-factor signalling where APS functions to inhibit mitogenesis [24,25].

Overexpression of JAK-2 in COS-7 cells increased basal JAK-2 phosphorylation and this was increased further by the

introduction of SH2-B β and led the authors to postulate that SH2-B may function as an intracellular ligand of the JAK-2 [17,26]. However, overexpression of APS or SH2-B alone did not change the basal IR autophosphorylation or phosphorylation status of other cellular proteins, but did increase the levels of ligand-stimulated phosphorylation. This indicates that this effect occurs principally via interactions with the activated receptor. Examination of the time course indicates that APS and SH2-B prolong IR autophosphorylation and the phosphorylation of its substrates following insulin stimulation.

The mechanism by which APS and SH2-B prolong IR phosphorylation is not clear. It is possible that APS and SH2-B stabilize the active conformation of the IR or compete with tyrosine phosphatase. PTPs such as PTP1B are known to dephosphorylate the IR at the activation loop [21,27]. It is possible that binding of either adapter to the receptor physically prevents phosphatase access or that these adapters may bind to PTPs themselves, preventing binding to the receptor. PTP1B is known to bind to the activation loop of the IR and regulate its function [21,27]. Other phosphoproteins, such as Grb10 and Grb14, also bind to the activation loop of the IR [28], but these have a negative effect on receptor signalling [29], indicating that simple competition is unlikely to be the basis of this effect. It has been shown that APS and SH2-B augment Trk receptor signalling, possibly as a result of multimerization of these proteins [30]. APS and SH2-B may cluster activated receptors and thereby prolong signalling in this way. Changes in the activity and expression of tyrosine phosphatases do not appear to explain this effect. In fact, we found a slight increase in the expression of PTP1B, which would be more likely to accelerate dephosphorylation of proteins.

We examined the role that APS and SH2-B have in the conversion of the IR's tyrosine kinase signals into serine/threonine kinase signalling via the Ras/Raf/MEK/ERK signalling pathway. In control cells, following acute insulin stimulation, ERK phosphorylation had returned to the basal levels by 30 min. In contrast, APS- and SH2-B-overexpressing cells showed prolonged and increased ERK activation and no decline in ERK phosphorylation, even after 60 min. The prolonged activation of ERK correlates with the prolonged IR activation, and may account for the observed ERK activation. However, unlike the IR phosphorylation, there was no decline in ERK phosphorylation. Insulin stimulation results in transient ERK activation that declines over time, even in the presence of continuous insulin stimulation [7,16,18]. At present, the process by which APS and SH2-B prolong ERK activation is not known, but may be a result of prolonged tyrosine phosphorylation. The proline-rich region of SH2-B α has been shown to associate constitutively with Grb2. It is also known that Shc binds to APS constitutively while Grb2 binds to APS in a phosphorylation-dependent manner [16]. On the other hand, Grb2 is constitutively associated through its SH3 domain to SOS and are in a complex as Grb2-SOS in the cytoplasm. This Grb2-SOS complex requires translocation to the plasma membrane where SOS can activate Ras. Dissociation of the Grb2-SOS complex by negative feedback is vital for the inactivation of Ras. Binding of Grb2 to APS and SH2-B may generate the APS-Grb2-SOS and SH2-B-Grb2-SOS complexes respectively, and these complexes may not be regulated by the negative-feedback mechanism. Phosphorylation-dependent association of Grb2 with APS may be competitive, since the Grb2 SH2 domain is also shown to bind the phosphotyrosine motif of SHC and IRS. Therefore, in addition to SHC and IRS proteins, APS and SH2-B are competing for the limited cellular pool of Grb2 [31]. The two SH2 domains of Grb2 can form alternative complexes with APS, SH2-B, SHC and IRS

proteins. Interaction of Grb2 through its SH2 domain with APS prevents it from interacting with IRS and SHC simultaneously, whereas Grb2 that is associated constitutively with SH2-B can still potentially interact with SHC and IRS proteins via its SH2 domain. This raises the possibility that SH2-B and APS are generating different signalling complexes following insulin stimulation without affecting their mutual functions, and this requires further investigation.

Similar effects were seen when PI3K-dependent signalling was analysed. Using acute insulin stimulation, detectable differences in the activation of Akt were found between CHO.T-APS, CHO.T-SH2-B and the control CHO.T-Hygro cells. These results indicate that, as well as increasing ERK activation, APS and SH2-B also increase Akt activation in CHO.T cells. The mechanism by which APS and SH2-B enhance Akt activation would presumably be an indirect result of the enhancement of proximal signalling events and these effects may be potentially anti-apoptotic [6,32].

The high sequence similarity in the SH2 domain of APS and SH2-B enables them to bind similar phosphotyrosine motifs. However, differences in their expression pattern and level of insulin-induced phosphorylation suggest that they may have distinct signalling functions. Indeed, SH2B has been shown to be required for reproduction [33]. Despite their structural similarity, they display markedly different propensities to serve as substrates for the IR. Stimulation of cells with a relatively low concentration of insulin was sufficient to induce APS phosphorylation, whereas SH2-B required a high concentration of insulin to stimulate its phosphorylation. Increasing the levels of SH2-B did not increase its phosphorylation by insulin. Both high and low levels of SH2-B-expressing cells show similar levels of insulin-induced tyrosine phosphorylation, suggesting that there may be an essential structural feature of SH2-B restricting its phosphorylation. This is in contrast with the Trk receptor where both proteins appear to function equally well as substrates.

The amino acid sequence of APS and SH2-B differ considerably in the PH domain that targets proteins to the cell membrane. The ability of APS and SH2-B to translocate to the plasma membrane in response to insulin may be determined by their PH domains and this may influence the efficiency of phosphorylation by the IR. Mutation of the PH domain abolishes insulin-stimulated phosphorylation of APS [11]. Similar precedents have been observed with other substrates including the IRS proteins [34] and DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositides) [35] where the PH domain facilitates coupling to the IR in intact cells. Indeed, some evidence to support this is seen using immunofluorescence where APS localizes to the membrane more efficiently than SH2-B. The targeting of proteins to the plasma membrane by PH domains depends on its ability to interact with specific phospholipids [36]. The PH domain of APS and SH2-B may preferentially bind different phospholipids. Therefore, differences in the phosphorylation may depend on lipid binding and/or their ability to translocate from cytoplasm to the plasma membrane. However, it appears that binding to the activation loop is sufficient to enhance IR autophosphorylation and kinase activity independently of phosphorylation. The putative role of the PH domain of APS could be addressed in future studies using PH-domain mutants or 'domain-swapping' experiments to determine whether SH2-B can be converted into an efficient substrate by replacing the PH domain. Interestingly, APS and SH2-B appear to function equally well as substrates for the Trk receptor [30] and augment signalling by dimerization. A similar mechanism may operate in the context of IR signalling.

In summary, we show that both APS and SH2-B can augment IR signalling by interacting with the IR activation loop. This is

in contrast with previously described activation-loop binding proteins. Both display distinct differences in phosphorylation and translocation in response to insulin, but have broadly similar effects in enhancing signalling. An understanding of the precise molecular basis for the interaction with the receptor may yield insight into strategies designed to modulate insulin signalling using activation loop binding proteins.

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